IMPROVED INOCULANT STRAINS OF *BRADYRHIZOBIUM JAPONICUM*

BACKGROUND OF THE INVENTION

Extensive testing of *B. japonicum* strains isolated from nodules of soybean plants has led to the selection of strains best suited for commercial production as inoculants. Inoculants are used in several forms such as mixed with peat as a carrier, coated on seeds or directly as a liquid inoculant. In soils where soybeans are routinely grown, the inoculant strain typically forms only a small number of the nodules on the roots of the soybean plant (e.g. Kvien *et al.,* 1981; Ellis *et al.,* 1984; Howle *et al.,* 1987). The indigenous soil strains form the bulk of the nodules. This is known as the problem of competition. The indigenous strains have unknown characteristics which give them an advantage in nodulation. Thus inoculant strains, whether natural or genetically enhanced for superior nitrogen fixation, do not substantially enhance plant productivity since they are incapable of forming the bulk of the nodules on the plant's roots. This invention addresses that limitation by having enhanced competitiveness due to mutation with transposon Tn5.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated strain of Bradyrhizobium having increased nodulation characteristics, wherein a gene comprising a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:2 under 5X SSC and 42°C wash conditions is not expressed. The strain may include a gene that may at least partially comprise SEQ ID NO:1 or SEQ ID NO:2. Preferably, the gene may comprise SEQ ID NO:1 and SEQ ID NO:2, wherein SEQ ID NO:1 is located upstream of SEQ ID NO:2.

The present invention is also directed to a strain of Bradyrhizobium having a deposit number of NRRL-B-30052 or NRRL-B-30053.

In another embodiment of the present invention, the invention is directed to a method for promoting nodulation of a leguminous plant, comprising:

(i) inoculating a plant or a place near a plant root with a nodulating effective amount of an inoculum comprising an isolated strain of Bradyrhizobium having increased nodulation characteristics, wherein a gene comprising a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:2 under 5X SSC and 42°C wash conditions is not expressed, and

(ii) allowing the Bradyrhizobium strain to inoculate the root.

In this method the leguminous plant may be soybean, cowpea mungbean or siratro.

In another embodiment of the invention, the invention is also directed to an isolated gene comprising a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:2 under 5X SSC and 42°C wash conditions. The gene may comprise at least in part SEQ ID NO:1 or SEQ ID NO:2. The gene also may comprise SEQ ID NO:1 and SEQ ID NO:2, wherein SEQ ID NO:1 is located upstream of SEQ ID NO:2.

The invention is also directed to a plant seed coated with any of the Bradyrhizobium strain discussed above.

In another embodiment, the invention is directed to a composition comprising an isolated strain of Bradyrhizobium having increased nodulation characteristics, wherein a gene comprising a nucleotide sequence that hybridizes to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:2 under 5X SSC and 42°C wash conditions is not expressed, and an agriculturally acceptable carrier thereof.

These and other objects of the invention will be more fully understood from the following description of the invention, the referenced drawings attached hereto and the claims appended hereto.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will become more fully understood from the detailed description given hereinbelow, and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein;

Fig. 1 shows nodule occupancy of I-110 ARS and BJ5019 when mixed together with varying proportions of USDA 438 and inoculated onto soybean plants.

Fig. 2 shows nodule Occupancy of BJ5019 when mixed with other B. *japonicum* strains at a ratio of 1:100 (BJ5019:other strain).

Fig. 3 shows nodule Occupancy of BJ5019 on other host plants in competition with I-110 ARS.

Fig. 4 shows the flanking regions of the Tn5 insertion site (SEQ ID NOS:1 and 2).

Table 1 shows nodulation of soybeans by mixtures of BJ5019 and TA11 Nod^+

Table 2 shows nodulation of soybeans by mixtures of BJ5019 and *B. japonicum* strain 119

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "genetic element" may be any nucleotide sequence that affects the expression of a particular gene or phenotype. A gene may be a genetic element.

A gene is a unit of nucleotide sequence that encodes a protein. In the invention, the gene is at least partly comprised of SEQ ID NO:1 or SEQ ID NO:2, or both. The gene may also comprise SEQ ID NO:1 and SEQ ID NO:2, wherein SEQ ID NO:1 is upstream of SEQ ID NO:2. Moreover, SEQ ID NO:1 and SEQ ID NO:2 may be contiguous. Preferably, this gene is derived from Bradyhizobium. Preferably, the Bradyrizobium is *Bradyrhizobium elkanii* or *Bradyrhizobium japonicum*. Most preferably, the gene is derived from *B. japonicum*.

It is also understood that the gene that hybridizes to SEQ ID NO:1 and/or SEQ ID NO:2 may be varied, as certain non-functional variations are possible without affecting the activity of the gene. Thus, the gene may contain sequences that preferably hybridize to the SEQ ID NO:1 or SEQ ID NO:2 or both in either contiguous or individual form, under 5X SSC and 42°C wash conditions.

In the present invention, the genetic element may be mutated. Preferably, the mutation may be made so that the gene is either not expressed, which is sometimes termed knock-out mutant, or expressed in low amounts. The mutation may be caused by chemical means such as EMS or MMS mutagenesis, or by sited directed mutagenesis such as by insertion of a disrupting genetic sequence.

Other methods include the insertion of transposons or insertion sequences. The insertion sequence may contain a sequence that codes for a selective marker, such as an antibiotic.

In the mid-western soybean growing areas of the U.S., serocluster 123 strains of *B. japonicum* (comprised of serogroups 123, 127 and 129 strains) are the dominant indigenous strains and are highly competitive. USDA 438 is a representative of serocluster 123. The nodule occupancy of BJ5019 and I-110 ARS when mixed with various proportions of USDA 438 is shown in Fig. 1. I-110 ARS is a derivative of USDA 110 which was selected for spontaneous resistance to azide, rifampin and streptomycin (USDA/ARS *Rhizobium* Germplasm Resource Collection) and is useful as a substitute for USDA 110 because it is easily distinguished due to the strain's resistance to those compounds. The number of BJ5019 cells required for 50% nodule occupancy is more than 100-fold less than with I-110 ARS; and for 25% nodule occupancy is almost 1000-fold less. Thus, in this type of experiment, BJ5019 is markedly enhanced for competitiveness relative to a derivative of the parent strain.

That BJ5019 is enhanced in competitiveness with other *B. japonicum* strains is illustrated in Fig. 2. In this figure, the results are shown from an experiment where BJ5019 was mixed at a ratio of 1:100 with a representative of other serogroup strains. It is apparent that BJ5019 is enhanced in competitiveness with all the strains tested.

Two *B. japonicum strains,* 119 (Patent No. 4,863,866), and TA11 Nod^+ (Patent No. 5,021,076) are claimed to be enhanced in nodulation and/or nitrogen fixation. Both of these strains were derived from USDA 110. Tables 1 and 2 illustrate that BJ5019 is superior to these strains in nodulation ability at a ratio of 1:1 in the inoculum; BJ5019 formed 81% of the nodules on soybean when mixed with strain 119 (U.S. Patent No. 4,863,866 herein incorporated by reference) (Table 2) and 90% of the nodules when mixed with TA11 Nod^+ (U.S. Patent No. 5,021,076 herein incorporated by reference)(Table 1).

Table 1. Nodulation of soybeans by mixture of *B. japonicum* strains BJ5019 and TA11 Nod^+ (U.S. Patent No. 5,021,076)

Ratio of strains in the inoculum		**Ratio of strains in the nodules**		
TA11 Nod^+	BJ5019	TA11 Nod^+	BJ5019	Both
1	1	0	90	10
10	1	23	65	12
100	1	73	23	4

The antibiotic resistance markers on strain TA11 Nod^+ are rifampicin, streptomycin, and azide. Strain BJ5019 is resistant to kanamycin and streptomycin. Nodule occupancy by BJ5019 was obtained by scoring growth on media containing kanamycin, while occupancy by TA11 Nod^+ was determined by plating bacteroids isolated from nodules on rifampicin containing media.

Table 2. Nodulation of soybeans by mixture of *B. japonicum* strains 119 (U.S. Patent No. 4,863,866) and BJ5019

Ratio of strains in the inoculum		**Ratio of strains in the nodules**		
Strain 119	BJ5019	Strain 119	BJ5019	Both
1	1	nd	81	nd
10	1	nd	40	nd
100	1	nd	8	nd

nd, not determined.

The antibiotic marker on strain 119 is kanamycin resistant. Strain BJ5019 is resistant to kanamycin and streptomycin. Nodule occupancy by BJ5019 was obtained by scoring growth on media containing kanamycin. Independent analysis of nodule occupancy by strain 119 was not technically possible due to limitations of antibiotic marker selection and serotyping (both strains belong to the same serogroup, e.g., USDA 110).

B. japonicum nodulates other legume plants in addition to soybean. That the enhanced competitive nodulation phenotype is not specific to soybean is illustrated in Fig. 3. When co-inoculated onto cowpea, mungbean and siratro, BJ5019 formed more than 80% of the nodules on these legumes when mixed at a 100:1 ratio (I-110ARS: BJ5019).

The foregoing demonstrates that new mutant strains of *B. japonicum, obtained* after transposon Tn5 mutagenesis, nodulates soybeans and other host legume plants significantly better than the parent strain and many other *B. japonicum* strains, when co-inoculated with these other strains.

In addition to inoculating the soil, a composition containing the Tn5 mutagenized strain of *B. japonicum* of the invention may be used to coat seeds. Coating is accomplished by immersing seeds in a *B. japonicum*-transposon Tn5 enriched solution.

The following examples are offered by way of illustration of the present invention, and not by way of limitation.

EXAMPLES

Example 1 - *Mutagenesis of USDA 110. Bradyrhizobium japonicum* strain USDA 110 was isolated from a soybean nodule and is included in a germplasm collection, USDA-ARS National *Rhizobium* Germplasm Collection, Beltsville, MD 20705. It has been widely used in research and in commercial inocula.

B. japonicum USDA 110 was mutagenized with Transposon-5 (Tn5). Transposons are naturally occurring genetic elements carrying antibiotic resistance genes. Transposon Tn5 has

genes which carry resistance to kanamycin and streptomycin. Tn5 mutagenesis of USDA 110 was performed by triparental mating as described by Ditta (1986). Individual colonies of USDA 110 harboring Tn5 were selected on media containing kanamycin and streptomycin (at 200 μg/ml) (Bhagwat *et al.*, 1991).

Example 2 - *Selection of competitive nodulation phenotype.* Tn5 mutants were screened for their competitive nodulation ability on the host plant *Glycine max* (soybean) cv. Williams. Nodulation assays were performed in sterile Leonard jar assemblies (Vincent, 1970). Soybean seeds were inoculated with a mixture of *B. japonicum* strains (various Tn5 mutants and strain Mc617, in the ratio of 1:100). The strain Mc617 is a Fix$^-$ mutant of USDA 110 (Bhagwat *et al,* 1991) and forms large numbers of ineffective nodules which are unable to fix N_2. Soybean plants were maintained on N-free nutrient solution for 4 weeks in a greenhouse before harvesting for nodule occupancy analysis (Bhagwat *et al.*, 1996). Surface sterilized nodules were examined for the presence of kanamycin and streptomycin resistant bacteria to determine competitiveness of Tn5 mutant strains. The Tn5 mutant which formed the greatest number of nodules (BJ5009) was selected. This strain was deposited with the Agricultural Research Service Culture Collection (NRRL) under provisions of the Budapest treaty and assigned NRRL No. B-30052. All restrictions imposed by the depositor on the availability to the public of B-30052 will be irrevocably removed upon granting the patent.

Example 3 - *Confirmation that the phenotype was due to the* Tn5 *insertion.* The selected mutant strain BJ5009 was confirmed to carry a single copy of Tn5 and the vector sequences were absent. The genomic DNA of BJ5009 flanking the Tn5 insertion was cloned at the *Eco*RI site into a suicidal vector pSUP202. The resultant plasmid was introduced into USDA 110 by triparental mating (Ditta, 1986) and exconjugants resistant to kanamycin and streptomycin (Tn5 markers) but sensitive to tetracycline (pSUP202 marker) were selected. The selected exconjugants were screened to confirm the Tn5 insertion into an identical *Eco*RI fragment as observed in BJ5009 and one such exconjugant was designated as BJ5019. This strain was deposited with the Agricultural Research Service Culture Collection (NRRL) under provisions of the Budapest

treaty and assigned NRRL No. B-30053. All restrictions imposed by the depositor on the availability to the public of B-30053 will be irrevocably removed upon granting the patent.

The strain BJ5019 was indistinguishable in competitive nodulation phenotype BJ5099.

Example 4 - *Cloning of the full-length Bradyrhizobium gene that hybridizes to SEQ ID NO:1 or SEQ ID NO:2.* The cloning of the full-length gene for which the nucleotide sequence of SEQ ID NO:1 and SEQ ID NO:2 may be a part, is accomplished using well-known molecular cloning methods, such as disclosed in for instance, Sambrook et al., which is incorporated herein by reference especially with respect to the cloning the full-length gene by hybridization to known nucleotide sequences.

Literature cited by authors before and hereinafter is more fully described below: The following is herein incorporated by reference:

Literature Cited

U.S. Patent Documents

4,863,866 9/1989	Zablotowicz et al.	435/172.1
5,021,076 6/1991	Kuykendall et al.	435/252.2

Other Publication

The following references are herein incorporated by reference:

Bhagwat, A.A., K.C. Gross, R.E. Tully and D.L. Keister (1996). J. Bacteriol. 178:4635-4642.

Bhagwat, A.A., R.E. Tully and D.L. Keister (1991). Appl. Environ. Microbiol. 57:3496-3501.

Ditta, G. (1986). Meth. Enzymol. 118:519-528.

Ellis, W.R., G.E. Ham and E.L. Schmidt (1984). Agron. J. 76:573-576.

Howle, P.K.W., E.R. Shipe and H.D. Skipper (1987). Agron. J. 79:595-598.

Kuykendall, L.D. and D.F. Weber (1978). Appl. Environ. Microbiol. 36:915-919

Kvien, C.S., G.E. Ham and J.W. Lambert (1981). Agron. J. 76:573-576.

Vincent, J.M. (1970). A manual for the practical study of the root nodule bacteria. IPB handbook No. 15. Blackwell Scientific Publications, Oxford, U.K.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second edition (New York, Cold Spring Harbor Laboratory Press)

It will be recognized that the invention is not limited to any particular theory or explanation as to the manner in which the mutation functions, the scope of the invention being defined in the following claims wherein: